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Distinction of the Two Type I Coppers in Bovine Ceruloplasmin

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Abstract

Redox potentials of the two type I copper ions, 'blue copper ions', of bovine ceruloplasmin (ferroxidase, iron(II): oxygen oxidoreductase, EC 1.16.3.1) were determined to be 370 and 390 mV (*vs.* NHE). These two type I copper ions were clearly differentiated during the anaerobic reduction process of oxidized ceruloplasmin and the reoxidation process of completely reduced ceruloplasmin by using absorption, circular dichroic and electron paramagnetic resonance spectroscopies. One of the blue copper ions is reduced faster and reoxidized very slowly, and is assumed to be located away from the active site of ceruloplasmin. On the other hand, the other blue copper ion, which is reduced more slowly and reoxidized rapidly, is supposed to interact with other types of coppers, such as type II (non-blue) and type III (EPR undetectable) coppers. The active site of ceruloplasmin is considered to be comprised of one type I, one type II and a pair of type III copper ions.

Introduction

Ceruloplasmin (ferroxidase, iron(II): oxygen oxidoreductase, EC 1.16.3.1) is a copper-containing oxidase which catalyzes the oxidation of Fe^{2+}, certain diamines and polyphenols, and furnishes electrons to dioxygen as the final electron acceptor [1]. Ceruloplasmin has two type I (blue), one type II (non-blue) and a pair of type III (EPR undetectable) coppers, and an extra cuprous ion. Generally, type I copper exhibits a strong absorption band with the typical maximum at around 600 nm; it gives an unusually narrow hyperfine splitting on its EPR spectrum because of the participation of a Cu–S (Cys) bond and a highly tetrahedrally-distorted tetragonal structure around the copper(II) ion. In addition, the reduction potential of type I copper is usually high. The paramagnetic type II copper gives a wider hyperfine splitting on the EPR spectrum and does not afford any prominent absorption bands in the visible region. Type III copper is EPR silent, since the two copper(II) ions are strongly antiferromagnetically coupled. However, the coupled cupric ions exhibit a rather strong absorption band at around 330 nm which decays upon acceptance of electrons from type I and II copper ions [2]. Ceruloplasmin is classified as a multicopper oxidase, together with laccase and ascorbate oxidase. Laccase contains one type I, one type II and two type III copper ions [3] and ascorbate oxidase contains three type I, one type II and four type III copper ions [4]. The number sets of each type of copper vary with the kind of multicopper oxidases.

Here we determined the reduction potentials of the two type I coppers of bovine ceruloplasmin and studied spectrometrically the reduction and reoxidation properties of each type of copper. The two type I coppers were clearly differentiated from each other, and it was found that only one of them interacts with type II and III coppers to form an electron-transfer chain.

Experimental

Ceruloplasmin was purified from bovine serum according to the method of Calabrese *et al.* [5], with the addition of a gel filtration step using Sephadex G-100. The final absorption ratio of A_{614}/A_{280} reached 0.045, insuring the purity of the enzyme. All reagents used were of the highest grade commercially available and were used without further purification.

Absorption spectra were measured on a Union SM-401 high-sensitivity spectrophotometer and on a Hitachi Model 323 spectrophotometer at room temperature. Circular dichroic spectra were obtained on a JASCO J-500A spectropolarimeter attached to a data processor DP-500N. EPR spectra were measured with a JEOL FE-1X spectrometer at 77 K. The amount of EPR detectable copper(II) ion was determined by the double integration method using copper(II) ethylenediaminetetraacetate as a standard. Total copper content in the enzyme was determined by using a Nippon-Jarrel Ash AA-1 atomic absorption spectrometer.

A 8 mm path length quartz cell attached to a three-way stopcock at its head was used for optical

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measurements in order to avoid oxygen. Aliquots of samples were withdrawn from the cell using a syringe to measure EPR spectra. Redox potentials of the two type I copper ions were determined spectrophotometrically at 614 nm. An Orion 801A digital ion-analyzer was employed to measure the potentials between a calomel electrode (0.1 M KCl) and a platinum electrode under argon atmosphere. Aliquots of reducing agent were added to the titrate solution through a Hamilton gas-tight syringe. Octacyanotungstate(IV) was synthesized according to literature methods to be used as a mediator [6].

Results and Discussion

Redox potentials of the two type I coppers of bovine ceruloplasmin were determined using hexacyanoferrate(III) and octacyanotungstate(IV) as mediators by spectrophotometric titration, according to a previously described procedure whereby the redox potentials of the two type I coppers of human ceruloplasmin were determined [7]. The resulting Nernst plot is given in Fig. 1. The plot was

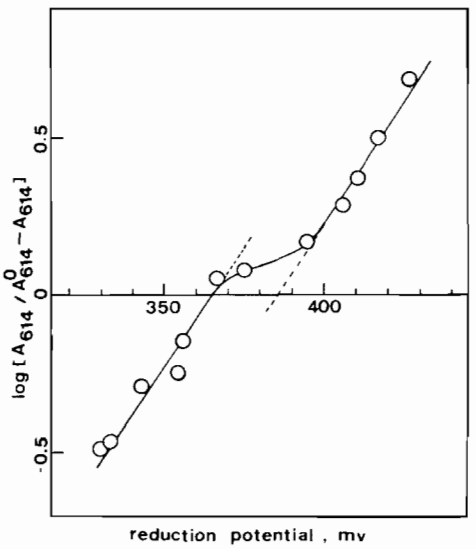

Fig. 1. Potentiometric titration of ceruloplasmin (12 μM per protein) with ascorbate at pH 6.0 and room temperature (21 °C) in 0.1 M sodium phosphate buffer under argon.

almost linear at both ends, giving slopes of $n = 0.91$ and half-wave potentials of $E_o' = 370$ mV (*vs.* NHE) and $n = 0.92$ and $E_o' = 390$ mV. These reduction potentials are considerably lower than those of human ceruloplasmin ($E_o' = 490$ and 580 mV) [7], but are common values for the most blue copper proteins (180–770 mV) [8]. Three type I coppers of cucumber ascorbate oxidase do not exhibit either the same redox potential ($E_o' = 350$ mV) or the same spectral properties [9]. Besides their differing redox

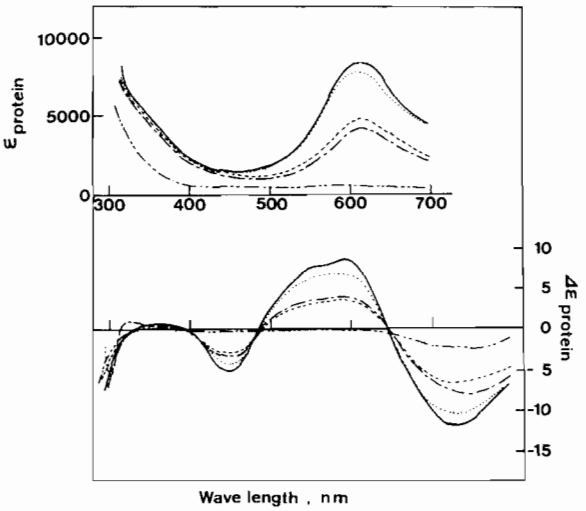

Fig. 2. Absorption and circular dichroic spectra of ceruloplasmin (19 μM) during the anaerobic reduction process with ascorbate (5.5 reducing equivalents/mol of protein) and the reoxidation process by air (21 °C, pH 6.0): native ceruloplasmin (——); 10 min after reaction with ascorbate (------); 160 min after reaction with ascorbate (– – –); soon after introduction of air to the reduced ceruloplasmin (– · –); *ca.* 1000 min after the start of reoxidation (· · · · · ·).

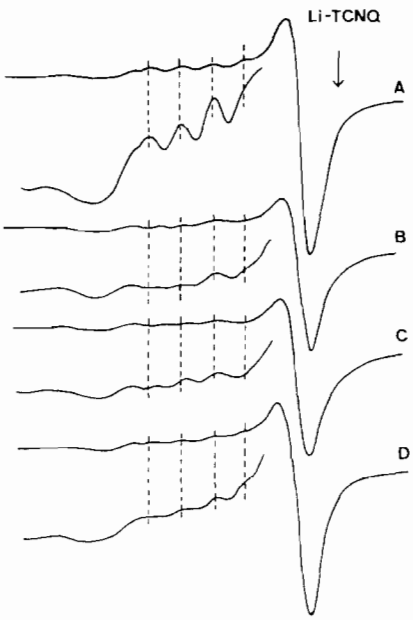

Fig. 3. EPR spectra of ceruloplasmin (19 μM) during the anaerobic reduction process with ascorbate and the reoxidation process by air (77 K): (a) native ceruloplasmin; (b) 10 min after reaction with ascorbate (Cu^{2+}, 28 μM); (c) soon after introduction of air to the reduced ceruloplasmin (Cu^{2+}, 41 μM); (d) *ca.* 1000 min after the start of reoxidation (Cu^{2+}, 61 μM).

potentials, the two type I coppers in ceruloplasmin afford EPR signals that are different from each other (*vide infra*).

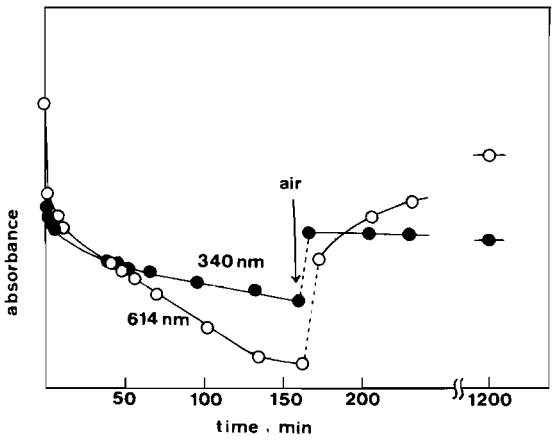

Fig. 4. The time course of absorption changes at 614 and 340 nm during the anaerobic reduction and the reoxidation of ceruloplasmin.

Anaerobic reductions of ceruloplasmin with ascorbate were followed by measurements of the absorption, circular dichroic (Fig. 2) and EPR spectra (Fig. 3). Absorption changes at 614 nm and 340 nm are presented in Fig. 4. The former comes from the type I copper(II) ions and the latter from the type III copper(II) ions. Upon reduction, the absorbance at 614 nm was lowered to 50% of the original value in *ca.* 20 min. There was also a simultaneous lowering of the absorbance at 340 nm; a 50% decrease was attained in *ca.* 25 min. The absorbance decrease was completed in *ca.* 160 min, giving rise to a reduced ceruloplasmin with no apparent band from the near ultraviolet to the visible regions. Soon after introduction of a small amount of air through a syringe, the absorbance at 340 nm regained the original intensity, while the absorbance at 614 nm increased to half of the original one. The intensity of this band continued to increase gradually, finally reaching about 90% of the original intensity after 1 day. All of the bands in the region of 430–800 nm recovered to half of the original intensities. Although it has been reported that only one of type I coppers contributes to the band at 450 nm [10], we found that both type I coppers seemed to contribute equally to this band. The EPR measurements indicated that the two type I copper ions are neither reduced nor reoxidized at the same rate. The type I copper ion whose spin Hamiltonian parameters are $g_{\parallel} = 2.22$ and $A_{\parallel} = 73$ G (broken lines in Fig. 4) was reduced faster. The other type I copper, which seems to have EPR parameters of $g_{\parallel} = 2.25$ and $A_{\parallel} = 64$ G, was reduced at a slower rate (Fig. 3b). At the beginning of the reoxidation of the reduced ceruloplasmin, an EPR spectrum corresponding to the superposition of the latter type I and the type II copper ions was obtained (Fig. 3c). (EPR parameters of the two type I coppers of human ceruloplasmin were evaluated to be $g_{\parallel} = 2.22$, $A_{\parallel} = 92$ G and $g_{\parallel} = 2.21$, $A_{\parallel} = 72$ G by a simulation study [11]. The latter values seem very near to those of one type I copper of bovine ceruloplasmin, but the former differ considerably from those of the other type I copper of bovine ceruloplasmin. Computer simulation study is now underway.)

It has been repeatedly reported that two of the type I copper ions are reoxidized at different rates; the faster one was called Cu(A) and the slower one Cu(B) [12]. It seems apparent that Cu(B) is reduced faster and reoxidized slowly. On the other hand, Cu(A) is reduced at a slower rate and reoxidized quickly together with type II and type III copper ions. This indicates that Cu(A) is assembled with type II and type III copper ions, constituting an electron-transfer chain. However, Cu(B) is independent of the active site of ceruloplasmin. Accordingly, ceruloplasmin contains the same set of different types of coppers at its active site as that of laccase. The main electron-transfer pathway of ascorbate oxidase appears in our recent experiment [13] to be constituted by the same copper set. The reason that Cu(A) is reduced more slowly than Cu(B) is that an electron is transferred to a type III copper ion and Cu(A) is oxidized again to accept another electron, as suggested by a stopped-flow experiment [14]. (An absorption change based on the intramolecular electron transfer between the type I and type II coppers in human ceruloplasmin was observed by use of a conventional spectrophotometer. However, a similar absorption change for bovine ceruloplasmin has never been obtained, even by use of a stopped-flow spectrometer.)

Recently, determination of the complete amino acid sequence of human ceruloplasmin was accomplished, and it was revealed that ceruloplasmin is composed of three-fold internal homologies [15]. Two of them contain portions homologous to simple blue copper proteins, plastocyanin [16], azurin [17] and plantacyanin [18]. In addition, one of them has the His–Cys–His sequence which is also found in laccase [19] and is believed to be indicative of a multicopper oxidase. The odd type I copper was considered to be a non-functional one. The same situation is believed to exist in bovine ceruloplasmin, although redox potentials and spectral properties of bovine ceruloplasmin are somewhat different from those of human ceruloplasmin.

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References

1 L. Lyden, in R. Lontie (ed.), 'Copper Proteins and Copper Enzymes', Vol. III, CRC Press, Boca Raton, Fl., 1984, p. 37.
2 L.-E. Andreasson and B. Reinhammar, *Biochim. Biophys. Acta, 568,* 145 (1979).
3 D. M. Dooley, J. Rawlings, J. H. Dawson, P. J. Stephens, L.-E. Andreasson, B. G. Malmstrom and H. B. Gray, *J. Am. Chem. Soc., 101,* 5038 (1979).
4 T. Nakamura, N. Makino and Y. Ogura, *J. Biochem. (Tokyo), 64,* 189 (1968).
5 L. Calabrese, F. Malatesta and D. Barra, *Biochem. J., 199,* 667 (1981).
6 J. Kleinberg (ed.), 'Inorganic Synthesis', Vol. VII, McGraw-Hill, New York, 1963, p. 142.
7 J. Deinum and T. Vanngard, *Biochim. Biophys. Acta,* 310, 321 (1973).
8 T. Sakurai, H. Okamoto, K. Kawahara and A. Nakahara, *FEBS Lett., 147,* 220 (1982).
9 K. Kawahara, S. Suzuki, T. Sakurai and A. Nakahara, *Arch. Biochem. Biophys., 241,* 179 (1985).
10 M. V. Chidambaram, A. Zgirski and E. Frieden, *J. Inorg. Biochem., 21,* 227 (1984).
11 P.-O. Gunnarsson, U. Nylen and G. Pettersson, *Eur. J. Biochem., 37,* 47 (1973).
12 R. J. Carrico, B. G. Malmstrom and T. Vanngard, *Eur. J. Biochem., 20,* 518 (1971).
13 T. Sakurai, S. Sawada, S. Suzuki and A. Nakahara, *Biochem. Biophys. Res. Commun., 131,* 647 (1985).
14 R. J. Carrico, B. G. Malmstrom and T. Vanngard, *Eur. J. Biochem., 22,* 127 (1971).
15 N. Takahashi, T. L. Ortel and F. W. Putnum, *Proc. Nat. Acad. Sci. U.S.A., 81,* 390 (1984).
16 P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris,J. A. M. Ramshaw and M. P. Venkatappa, *Nature (London), 272,* 319 (1978).
17 R. P. Amber, in A. Previero, J. F. Pechere and M. A. Coletti-Previero (eds.), 'Recent Developments in the Chemical Studies of Protein Structures', Inst. Nat. Sante Rech. Med., Paris, 1971.
18 M. Murata, G. S. Begg, F. Lambrou, B. Leslie, R. J. Simson, H. C. Freeman and F. J. Morgan, *Proc. Nat. Acad. Sci. U.S.A., 79,* 6434 (1982).
19 C. Briving, E.-K. Gandvik and P. O. Nyman, *Biochem. Biophys. Res. Commun., 93,* 454 (1980).